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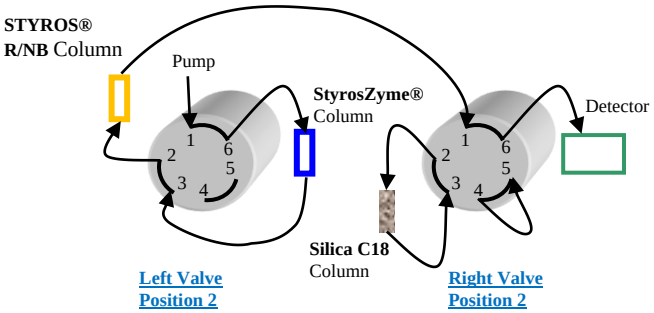
APPLICATION NOTE

Lysozyme Digestion.
TPCK Derivatized Compared with Non-Derivatized Trypsin in Digesting Lysozyme.

Using the setup that we have developed at OraChrom for automated digestion of proteins, Lysozyme was used to study additional factors of online digestion.
The peptides generated are more distinct in the resulting chromatogram and it is easier to notice the impact of any change and therefore realize the extent of control one has over the online digestion process as opposed to batch or similar mode of digestions.

The stability of the polymeric base matrix itself and the stability of the immobilized enzyme are key to rely on the results. No such phenomenon as autolysis and the like needs to be considered as we have made it clear in Application Notes 142 or 153 among others.

The following shows the starting position of the valves used:



Setup 1

In this first position, the StyrosZyme® enzyme column and the polymeric STYROS® R/NB column only, are online and the Silica C18 column is not exposed to any high pH aqueous buffer that is used for the digestion.
It is therefore possible to safely scout the right digestion buffer as well as the optimum conditions for the full digestion prior to the use of the silica column.
Both polymeric columns can tolerate high pH.

The buffers used are as follow:

Buffer A: 0.8 % TFA in H2O: ACN 98:2 (for mapping)
Buffer B: 0.8 % TFA in H2O: ACN 30:70 (for mapping)
Buffer C: 0.1 M Tris, pH = 8.5 (for digestion)

It is worthwhile to note that the columns used are all narrow bores of 2.1 mm or short normal bore of 4.6 mm of 5 cm.

Therefore, the solvent consumptions are low and multiple injections can be performed to verify the reproducibility of the injections as well as the stability of the immobilized enzyme column.

The columns include:

1. A polymeric reversed phase column **STYROS® R/NB** Simulated-Monolith™ 2.1x 50 mm
2. A normal bore polymeric column of 4.6 x 50 mm StyrosZyme® Trypsin
3. A normal bore polymeric column of 4.6 x 50 mm StyrosZyme® TPCK-Trypsin
4. A silica narrow bore column from Waters. Acquity UPLC BEH C18 1.7 um 2.1 x 50 mm.
5.

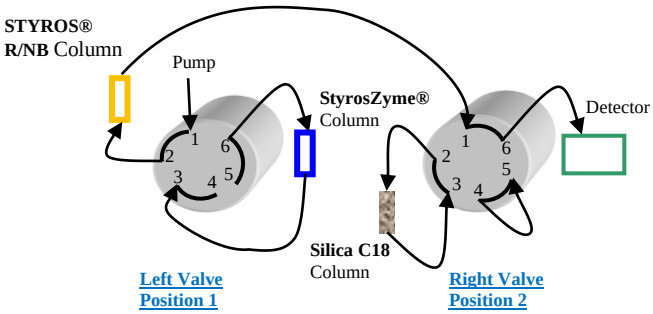
1-Equilbrate the enzyme column with all columns except the silica column, in line, as shown in Setup 1.

Time	% Of buffer C	Flow rate (ml/min)
0	100	0.2
0.01	100	0.2
5	100	0.2

2-With both columns in line as in Setup 1, 5 of a solution of 5 mg/ml Lysozyme in buffer A is injected, and the resulting digests are dumped on the polymeric reversed phase column using the following method:

Time	% Of buffer C	Flow rate (ml/min)
0	100	0.2
10	100	0.2

In the second setup, the left valve is switched to position 1 with only the STYROS® R reversed phase column online to equilibrate it with the starting gradient solvent.



Setup 2

3-The reversed phase Polymeric column only is now in line as in Setup 2 to wash the leftover salt and prepare it for the reversed phase mapping.
It is also ready for hyphenation with a mass spectrometer.

Time	% of buffer B	% of buffer A	Flow rate (ml/min)
0	5	95	0.2
1	5	95	0.2
10	5	95	0.2

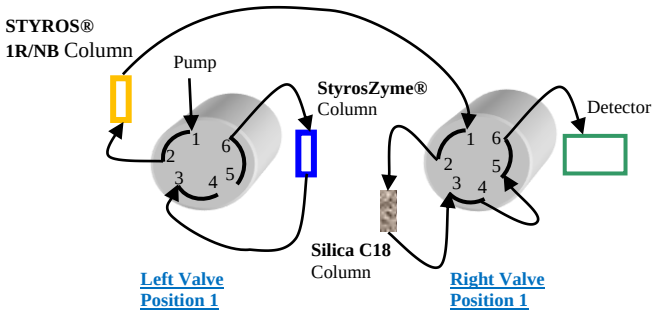
4-The digested peptides are now trapped on the polymeric reversed phase column and can be mapped following a gradient.
The setup is now as Setup 2.

Time	% of buffer B	% of buffer A	Flow rate (ml/min)
0	5	95	0.2
0.1	5	95	0.2
90	70	30	0.2

Once the conditions of the digestion are satisfactory, one can use the second switching valve to bring the Silica C18 column online as shown in Setup 3.
The same gradient as in step 4 can be used for the mapping to run the separation on the C18 Silica column in addition:

4'-The digested peptides trapped on the polymeric reversed phase column can now be mapped on the C18 Silica column in addition.
The setup is now as Setup 3.

Time	% of buffer B	% of buffer A	Flow rate (ml/min)
0	5	95	0.2
0.1	5	95	0.2
90	70	30	0.2



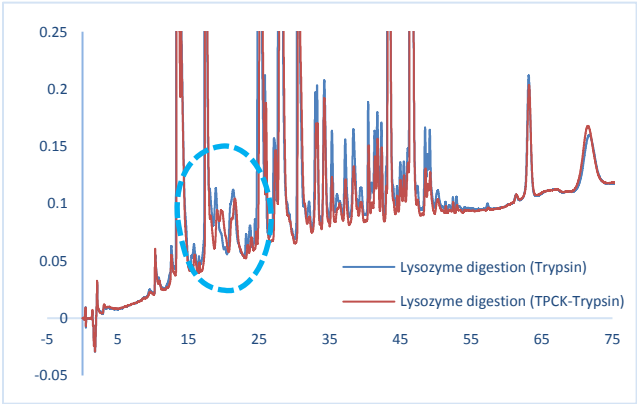
Setup 3

4''-In this step, using the same setup 3, the columns are washed with high organic to make sure they are devoid of any leftover proteinaceous species.

The setup remains Setup 3.

Time	% of buffer B	% of buffer A	Flow rate (ml/min)
0	5	95	0
0.01	60	40	0.2
1	100	0	0.2
2	100	0	0.2
4	5	95	0.2
8	5	95	0.2

The system is now ready for the next cycle to check the reproducibility of the digestion.
The temperature is set at 37°C for all sequences. The absorbance is monitored by the PDA at 214 nm.
Lysozyme is being digested on a normal bore column at a volumetric flow rate of 0.2 ml/min.
This would translate to a linear flow rate of 72 cm/hr. on an empty column.
The digestion is complete.
In the following chromatograms compare the Lysozyme digests on Trypsin compared with the digests on TPCK-Trypsin to avoid any Chymotryptic digestion.
As depicted here, aside from the peak intensities, there are some differences early on where less hydrophobic peptides elute.



With similar residency time, we can assess the effect of the derivatization of Trypsin to avoid any chymotryptic additional digests.
However, to fully deduct any additional information, one needs the result of a mass spectrometer and the specific protein digested.

Although the digestion goes to completion either at 0.2 ml/min or at 0.05 ml/min, these are linear flow rates of 72 and 18 cm/hr respectively, in both cases regardless of how brief the residency time is, it appears that Eddy's shear force is also a factor in these processes and can assist the process.

We have shown here two runs under the same conditions of the digestion of Lysozyme at low and high flow rates to highlight the level of control one is provided with the advantages a stable polymeric Simulated-Monolith™ with immobilized enzyme provides.
Furthermore, the use of small columns provides the possibility of running the process many times both the make sure it is reproducible, as well as the endurance of the enzyme reactor.